PONTIFICIA UNIVERSIDAD CATÓLICA DEL ECUADOR
FACULTAD DE CIENCIAS EXACTAS Y NATURALES
ESCUELA DE CIENCIAS BIOLÓGICAS

Characterization of pathogens related to *Helianthus annuus* cultivated in a production farm and screening of fungal inhibitory extracts

Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas

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Certifico que la disertación de Licenciatura en Ciencias Biológicas del candidato Berenice Salomé Benavides Benavides ha sido concluída de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Carolina Portero

Directora de la disertación

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“Porque mejor es la sabiduría que las piedras preciosas; Y todo cuanto se puede desear, no es de compararse con ella”.

Proverbios 8:11

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TÍTULO: Characterization of pathogens related to *Helianthus annuus* cultivated in a production farm and search of fungal anthagonists extracts

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Resumen

Apesar de que la producción de Girasol ha aumentado en el Ecuador, los registros de sus enfermedades estan incompletas y por consecuencia el conocimiento acerca de como controlarlas y/o tratarlas está limitando la expansión de este cultivo. Nuestro estudio se enfoca en aislar e identificar algunos de los patógenos de girasol así también de

terminar algunos tratamientos potenciales para el control de enfermedades usando extractos de plantas y de hongos endófitos con efectos inhibitorios. Nuestros resultados muestran que cuatro microorganismos aislados de plantas de Girasol enfermas, provenientes de una finca ecuatoriana productora-exportadora, son de hecho patógenos de girasol. Estos resultados son importantes para nuestro país dado que dos de los patógenos aislados (*Sclerotinia sclerotiorum*, *Pseudomonas cichorii*) no presentan registro en Ecuador. También se encontró cinco hongos endófitos potenciales que pueden ser usados para controlar de los patógenos de Girasol; ya sea en forma de extracto o como fuente rica en compuestos orgánicos volátiles.

Palabras clave: Girasol; Endofito; Antagonista;

Abstract

Sunflower production has increased in Ecuador, however, records of its diseases are incomplete and consequently the knowledge of how to control and/or cure is limiting sunflower crop expansion. Our study focused on isolating and identifying sunflower pathogens and screening for potential treatments using fungal endophytes and plant extracts with inhibitory effects. Our results show that four microorganisms isolated from diseased plants from one productive farm were indeed sunflower pathogens. These results are important to this country owing that two of them (*Sclerotinia sclerotiorum* and *Pseudomonas cichorii*) have not been reported in Ecuador. We also found five potential endophytic fungi that can be used against sunflower pathogens as extracts or as rich source of volatile organic compounds.

Keywords: Sunflower; Endophytes; Antagonists;

Introduction

Ecuador is one of the largest exporters of tropical flowers in the world (BCE 2011), however sunflower production does not meet local demand. In 2015, around 24,700 tons of sunflowers were imported, costing approximately $25,000 million USD (BCE 2016). This existing demand has increased given way to and increase in sunflower production in Ecuador (PROECUADOR 2013; Riera and Zuleta 2013).

Sunflower *(Helianthus annuus)* is a yearlong herbaceous plant native of North America (Blackman et al. 2011) that grows in temperate environments (Vollmann and Rajcan, 2009). Due to its multiple uses (Davey and Massod, 2010; Vollmann and Rajcan, 2009) it has been introduced to Europe, Africa and Asia since the 15th century (Davey and Jan 2010; Gielen 1992).

Sunflower was introduced as a crop in Ecuador in the 1980's and interest in its farming has progressively increased since 2009 (Andramunio and Haro 2009). Although this crop has been grown in Ecuador for several years, there is no official registry of pathogens that affect it nor their possible treatments; this lack of knowledge limits the maintenance and increase of sunflower production in Ecuador (Torres 2004).

Undoubtedly, the distribution of sunflower pathogens has followed the introduction of sunflowers in each continent (OECD 2006). There are at least 30 identified diseases caused by fungi, bacteria and viruses on cultivated sunflowers globally (NSA 2015). Some of these diseases represent significant economic loss, such as white rot, one of the most common sunflower disease caused by the fungus *Sclerotinia sclerotiorum* (McLoughlin et al. 1992) and also bacterial leaf spot disease which is caused by two cosmopolitan species of *Pseudomonas: P. syringae* and *P. cichorii*. These pathogens (*S. slerotiorum, P cichorii* and some pathovars of *P.syringae*) have not been reported in Ecuador (Agrocalidad 2012-Resolución Nº 0116), so it is important to identify the pathogens that could affect some crops in order to prevent diseases and determine adequate treatments.

Finding an adequate treatment represents a challenge, especially because current disease control depends on the use of chemical pesticides, which are hazardous for human and animal health and to the environment (Damalas and Hashemi 2010; WHO 2009). These chemicals also increase bacterial and fungal resistance (eg. *Sclerotinia sclerotiorum* to benzimidazole fungicide) (Gossen and Rimmer 2001). This has encouraged the quest for

alternatives to traditional pesticides (Mancini and Romanazzi 2013), such as plant and fungal-based extracts (Shilpa et al. 2015; Aslam et al. 2010).

Bioactive molecules identified and isolated from fungal endophytes and plants could be effective biocontrol agents as these are toxic to specific pathogens or can lessening disease severity (Arnold 2007; Narisawa et al. 1998; Wicklow 2005; Wink 1993; Abbas et al. 2016). Fungal endophytes are present in all plant families and colonize the host in an asymptomatic form (Arnold 2007; Khan et al. 2010).

The endophyte fungal collection (CEQCA) of the Pontificia Universidad Católica of Ecuador is a source of extracts that can be evaluated for potential bioactivity against pathogens (Rundel et al. 2015).

Our main objetive was identify pathogens that could affect sunflower farm production in Ecuador and to screen endophyte fungal extracts from the CEQCA and plant extracts, as sources of biomolecules with antibiotic and/or anti-fungal effects.

Materials and methods

Isolation of Pathogens

Isolation of Fungi and Bacteria from Helianthus annuus

Specimens were collected from five diseased sunflowers plants of the variety "Vincent Choice" from a local farm located in the Los Chillos valley, near Sangolqui city (Pichincha Province) [-0.032598319 latitude, -78.3802413 longitude at 2600 masl]. Plants sampled presented disease symptoms such as chlorotic spots on leaves and white mycelia growing on inflorescence and stem surfaces.

Isolation of fungi and bacteria was conducted under laboratory conditions, following a modified protocol described by Ismael and collaborators (2012). Root, stem and leaf surfaces were washed with 3% sodium hypochlorite followed by three washes with sterilized distilled water. Each disinfected tissue was macerated and after 20 min it was streaked, using a T- streak pattern, onto different media plates with either 1:10x Potato Dextrose Agar (PDA), Luria Bertani Agar (LB), 2% Malt extract or King B (KB) media. Plates were incubated at 27°C for 48 to 72 h.

Bacterial colonies were subcultured in KB media plates and incubated at 27°C, while fungi were isolated from terminal hyphae (Strobel et al. 2001) and incubated in PDA 1x. Pure cultures of bacteria and fungi were cryopreserved at -80°C (Muller et al. 2004; Bonavia et al. 2012).

Fungi and Bacteria Identification

Preliminary identification of isolated bacteria was performed at the "Centro de Investigación Microbiológica" (CIM) in Guayaquil-Ecuador by matrix-assisted laser desorption/ionization time of flight mass spectrometry method (MALDI-TOF-MS) using a Microflex LT MALDI-TOF spectrometer (Bruker Daltonics Inc., Bremen, Germany), which analyzes the protein profile of the 16s subunit rRNA (Seng et al. 2013). The recorded spectra score was compared with CIMs identification database (Cobo 2013). For taxonomic identification, criteria described by Santos et al (2013) were used.

Bacterial taxa determined as sunflower pathogens by MALDI-TOF were then identified by DNA sequencing. DNA extraction was done using a Wizard genomic DNA Purification Kit (Promega, Madison, WI, USA). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using 27F and 149R primers (Frank et al. 2008). The amplification protocol consisted of one cycle of initial denaturation for 1 min at 95ºC, 40 cycles of denaturation for 45s at 95°C, annealing for 30s at 54ºC and extension for 45s at 72ºC; and a final extension for 5 min at 72ºC.

Fungal DNA extraction was carried out from a 7 day old mycelium culture following a Chelex extraction protocol based on Bucheli et al. 2000 and Camacho et al. 1997. Mycelium was mixed with 5% Chelex and boiled at 100ºC for 10 min, then samples were centrifuged at 13000rpm for 2 minutes. Next, supernatants were boiled for 5 min, tubes were then put on ice and centrifuged again, and finally the DNA was precipitated.

For fungal identification the internal transcribed spacer (ITS) region of 5.8S rDNA gene using universal primers ITS1 and ITS4 (White et al. 1990) was amplified by PCR under conditions described by Bascom-Slack and collaborators (2012).

Amplified PCR products from fungi and bacteria were sent to Macrogen Sequencing Facilities (Seoul-Korea). Universal primers 518F and 800R (Lane 1991) were used to sequence bacteria and primers ITS 1and ITS 4 (White et al. 1990) for fungi.

A consesus sequence was obtained for each sample, the search for similar sequences was carried out using Basic Local Alignment Search Tool with nucleotide database (BLASTn) (Koski and Golding 2001). Subsequent phylogenetic trees were conducted. Consesus sequence from each sample were multi-aligned with the sequences that show the nearest hit and query cover on BLASTn using Muscle software (Edgar 2004). Alignment gaps were cleaned using Phyutility 0.5 Software (Posada 2008). Then, randomized accelerated maximum likelihood (RAxML) tool, applying the GTR model, was used to generate each phylogenetic tree (Stamatakis 2006) with 100 bootstrap replicates.

Three phylogenetic trees were made, one for each putative disease, *Chloroscypha enterchrom* was used as an out group for white rot disease (*Sclerotinia sclerotirum* phylogenetic tree), *Pleospora herbarum* for leaf spot (*Alternaria alternata* phylogenetic tree) and *Pseudomonas tremae* for bacterial leaf spot *(P. syringae – P.cichorii* phylogenetic trees) (Jeon et al. 2006; Chapman et al. 2012; Khodaei and Arzanlou 2013).

Pathogenicity Test

Koch Postulates were completed to corroborate that the isolated bacteria and fungi were pathogenic on sunflower plants.

Commercial sunflower seeds of the variety "Vincent Choice" were disinfected following the methods described by Schrammeijer 1990. To induce germination each seed was placed into small glass vials containing 20 mL of Murashige and Skoog medium (MS) (Nestares et al. 2002) and maintained in darkness for 3 days, after which the seeds were exposed to sunlight (Taski-Ajdukovic and Vasic 2005). To verify seed disinfection the isolation method described previously (Ismael et al.2012; Strobel et al. 2001) was used, in order to isolate any microorganism from the first successful germinated plant.

After 10 days of growth, twenty healthy axenic plants were placed in small pots with previously sterilized PRO-MIX FLX substrate. These plants were inoculated with the identified pathogens, two fungi and two bacteria. For bacterial inoculation, pure cultures were diluted in 0.85% saline solution and adjusted to 1×10^9 CFU/mL after which, 20 µL of the bacterial suspension was placed in a 5mm incision in the node of the oldest leaf (Rhodehamel and Durbin 1985). Fungal inoculation was done following the same bacteria protocol but using a suspension of 1 to 2×10^6 spores/ml (Benito et al. 1998; Khodaei and Arzanlou 2013). Individual plant growth chambers were assembled with plastic bags to

avoid external contamination and contact among the test organisms. Plants in the growth chambers were maintained at 36-38ºC, under sunlight condition and automated irrigation every two days. Finally, after 10-12 days of observation, isolation method described previously (Ismael et al.2012; Strobel et al. 2001) was used to reisolate the pathogens from the infected plant.

Disease severity index (DSI) was determined with the formula used by Kim et al. (2000) and a criteria scale from 0 to 6 as Maselli et al. (2000) (Table 1) including death of the specimen as criteria number six. Disease severity was recorded every three days for bacterial infection and every two days for fungal infection.

The area under disease progress curves (AUDPC) was calculated for all specimens, following the formula used by Kim et al. (2000), using the values of DSI and considering hours as the progress variant.

Evaluation of bioactivity against fungal and bacterial sunflower pathogens

In order to evaluate inhibitory activity over sunflower pathogens, two assays were performed: one with organic extracts from plants and fungal endophytes and another with volatile organic compounds from endophytic fungi. Extraction conditions are shown in Table 2.

Thirty three extracts from CEQCA dissolved in methanol were tested (Table 2). Agar direct diffusion method was performed in triplicate based on the protocols of Bascom-Slack (2012) and Document M02-A11 (Clinical and Laboratory Standards Institute 2012). PDA plates were divided into four quadrants: In two of the quadrants, 5µL of crude extracts were added twice, with an interval of 15 min between the additions. In the remaining quadrants one had no extract (negative control) and the other contained 5ul of methanol, a control to evaluate methanol effect on the pathogens.

In the case of bacterial pathogens, a 0.5 McFarland standard turbidity inoculum was streaked uniformly with a sterile cotton swab onto the dried and diffused extract spots and each plate was maintained at 27 ºC for 24h. The percentage of inhibition of fungal assays was determined according to the equation proposed by Nourozian et al. (2006). Total inhibition of bacteria was considered when a clear halo (showing no bacterial growth) was observed surrounding the extract spot; and partial inhibition when there was bacterial growth within the inhibition halo.

In the case of fungal pathogens, 3×3mm plugs were placed on the dried and diffused extract of each quadrant. Each plate was maintained at 37ºC and observed for three days.

Determination of antibacterial Minimal Inhibitory Concentrations (MICs)

MICs were determined by a microdilution assays for each of the fungal endophyte extracts that presented total inhibition (100%) in the plate bioassay. Fungal endophytes were grown in Potato Dextrose Broth (PDB) for two weeks and then filtered through a cheesecloth, the filtrate was extracted successively with dichloromethane (CH_2Cl_2) and ethilacetate (EtOAc), rotary evaporated, dissolved in methanol and preserved at 4°C in glass vials (Bascom-Slack 2012).

Crude extracts obtained were adjusted to a 10mg/mL concentration and eight twofold dilutions were made in triplicate (Clinical and Laboratory Standards Institute 2012). Bacterial suspension was adjusted to 0.5 McFarland turbidity standard ($1,5\times10^8$ CFU/ml) and finally diluted to 10^4 CFU/ml in Luria Bertani Broth medium (LB) (Katoch et al. 2014). For the experiment 96-well flatbed microplates were used; each well contained 10 µL of the bacterial suspension, 10 µL of extract dilution and 90 µL of LB medium (Arivudainambi et al. 2011). The assays included positive and negative controls (Clinical and Laboratory Standards Institute 2012). Spectrophotometric readings were taken by Microplate Manager® Bio-Rad Inc. 6 at 48h using a 490 nm filter (Pfaller et al. 1995).

Volatile Organic Compounds (VOCs) Effect on Sunflower Pathogens

Four endophytic fungi of the genus *Muscodor* were selected from the CEQCA (Table 2). Assays were performed following a modified protocol from Strobel et al. (2001). Bi-Petri plates were used for this assay, where a PDA plug containing *Muscodor* was placed in one side of the plate and after three days of fungal growth (Meshram et al. 2013) each one of the bacterial or fungal pathogens were placed in the other side. In the case of bacteria, 0.5 McFarland standard turbidity inoculum was streaked uniformly in Muller Hilton Agar (MHA) medium with a sterile hyssop. In the case of fungi a 3×3mm plug was placed in PDA medium (Banerjee et al. 2014).

Each experiment was performed in triplicate plus one control plate. Total inhibition of fungal pathogens was obtained when no growth was observed (100% inhibition) while partial inhibition was considered when pathogens presented less inhibition than the control

without exposure to VOC´s according to the equation given by Nourozian et al. (2006). For bacteria, total inhibition was only considered when no growth was recorded.

Seed treatment using antagonistic fungal endophytes in vitro

To use endophytic fungi as antagonists in sunflower farming these should be able to colonize the plant without harming it. In an initial plant colonization trial we used two endophytic fungi that showed total inhibition against sunflower pathogens in agar diffusion assays for colonization by seed treatment.

Sunflower seeds of the variety "Vincent Choice" were disinfected following the method described by Schrammeijer (1990). Conidial suspensions of four different concentrations (2×10^8, 2×10^6, 2×10^4 and 2×10^2 spores/ml) (Benito et al. 1998) were prepared from two endophytic fungi with potential inhibitory activity.

Twenty-four disinfected seeds were placed into each conidial suspension for at least 2h (Burgess and Hepworth 1996). Then, seeds were placed into small glass vials with 20 mL of MS (Nestares et al. 2002; Taski-Ajdukovic and Vasic 2005). Seed growth was observed for 10 days for colonization signs.

Results

Identification

Twenty strains of bacteria were isolated and identified using MALDI-TOF-MS technology (Table 3). According to the spectra score, identification of four bacterial genera (52A, 32A, 51B, 31A) was reliable, but species identification was probable. Identification for the remaining sixteen bacterial genera appears to be probable.

Two bacteria (42C, 43A), determined as sunflower pathogens by MALDI-TOF, were chosen for molecular identification. These were identified as *Pseudomonas syringae* (100% Identity, 99% Query cover) and *Pseudomonas cichorii* (100% Identity, 100% Query cover) according to the Basic local alignment search tool (BLAST) results and the Maximum likelihood analysis (Figure 1).

Using the same methodology, one of the four fungi isolated was identified as *Alternaria alternata* (100% Identity, 100% Query cover) (Figure 2) and three as *Sclerotinia sclerotiorum* (100% Identity, 100% Query cover) (Figure 3).

Pathogenicity Test

Koch's postulates were completed for all isolated sunflower pathogens. All plants inoculated with the pathogens showed chlorotic and necrotic lesions.

Plants infected by *P.syringae* and *P.cichorii* showed brown spots surrounded by a chlorotic halo, and some plants presented large irregular areas on the leaf edges that coalesced progressively from day 6 until necrosis of the tissue at day 10 (Figure 4G; 4H; 4I). Both species produced bacterial leaf spot symptoms.

Plants infected by *A. alternata* showed clear chlorotic lesions on leaves until day 6; on day 8 some lesions turned necrotic and white mycelia became visible on stem and leaf surfaces. The lesions first affected basal leaves and then expanded to the upper leaves; this symptomatology is consistent with leaf spot disease (Figure 4A; 4B; 4C). Plants infected by *S. sclerotiorum* showed white rot disease symptoms, where chlorotic and necrotic leaves were observed from day 3 and white mycelia was visible on day 6 after inoculation (Figure 4D; 4E; 4F).

The area under disease progress curve was 17388 for *P. syringae* and 12708 for *P. cichorii,* 12864 for *A. alternata,* and 11880 for *S. sclerotiorum* according to DSI (disease severity) (Figure 5).

Evaluation of bioactivity against sunflower pathogens

The inhibitory effect of endophytic fungi was studied under *in vitro* conditions. Data from 36 extracts tested (Table 4) showed that four extracts (CEQCA-G1396e2, CEQCA-G1396e1, CEQCA-O3215d2, CEQCA-O3215e2) presented total inhibition against both bacteria (*P. syringae* and *cichorii*). Three extracts, two from endophytic fungi and one from a plant, presented partial inhibition for one or both bacteria (Table 4) and the rest of extracts (80.5%) did not present total inhibition.

The same extracts were also tested against pathogenic fungi; four showed total inhibition against *S. sclerotiorum* (Table 4). One extract, CEQCA-G1396e2, showed total inhibition against *A. alternata.* Six extracts presented partial inhibition against one or both fungi. The rest of extracts, did not present inhibition.

The test with volatile organic compounds over sunflower pathogens showed that two *Muscodor* fungi (CEQCA-O0003, CEQCA-O0224) suppressed the growth of *P. syringae*

and *S. sclerotiorum* (total inhibition). Additionally, *Muscodor* fungi (CEQCA-O1100) suppressed the growth of *S. sclerotiorum.*

Minimal Inhibitory Concentration (MIC)

Microdilution assays showed a minimal inhibitory concentration of 10mg/mL against both bacteria for the extracts CEQCA-G1396e2 and CEQCA-O3215d2.

Minimal inhibitory concentrations for CEQCA-O3215e2 extract was 5 mg/mL against *P. syringae* and 2.5 mg/mL against *P.cichorii*.

In vitro seed treatment using antagonistic endophytic fungi.

All seeds placed on CEQCA-G1396 fungal dilutions showed necrotic spot on the cotyledon during the first three days of the experiment and all germinated seeds showed distortion on the leaves. The presence of mycelia varied according to the dilution. Dilution 2×10^{-2} presented mycelia at day ten; dilution 2×10^{-8} presented mycelia at day six, and the rest of the dilutions presented white mycelia at day nine (Figure 6).

Seeds placed on CEQCA-O3215 fungal dilutions showed brown spots on some seeds and delayed germination compared with the control seeds. After day 3, all germinated seeds showed growth of white mycelia (Figure 6).

Discussion

Four microorganisms isolated from diseased sunflower plants were isolated and identified as sunflower pathogens by molecular techniques and confirmed using Koch Postulates. Also we obtained inhibitory activities from nine endophyte extracts, two plant extracts and three *Muscodor* fungi.

Identification through MALDI-TOF is widely used for screening of routine samples for bacterial identification due to its low costs and quick results. Even though this method is known to be accurate and sensitive (Croxatto 2012), identification could be affected by systematic instrument errors (Tracy et al. 2009) and also limited by the database used (Cobo 2013). Despite low spectra scores were obtained for most of the isolates (Table 3), it was useful in this research to screen all isolated bacteria in a short time. Molecular identification was needed to identify isolated fungal pathogens and also to confirm species of bacteria considered as sunflower pathogens by previous MALDI-TOF identification.

In the case of bacteria molecular identification the 16S gene rRNA was used. This gene is the most common housekeeping genetic marker used in bacterial identification. Its present universally among bacteria and includes regions with species-specific variability which makes genus or specie identification possible (Mignard and Flandrois 2006). Its large enough for bioinformatic purposes and its function has not changed over time, an important fact given that random sequence changes are more accurate for evolutionary studies and conserved sequences are more accurate for identification (Janda and Abbot 2007). Meanwhile, the 5.8S gene rDNA and the internal transcribed spacer (ITS) region was used for fungi molecular identification because this gene is also highly conserved and its ITS regions are divergent and distinctive with the highest probability of successful identification for a extensive range of fungi (Schoch et al. 2012; Ferrer et al. 2001).

Pathogenicity tests indicated that the isolated organisms from diseased tissue are indeed sunflower pathogens, and all four Koch postulates were completed. Pathogen identity were also confirmed by means of disease symptomatology. *Pseudomonas syringae* and *P. cichorii,* the causal agents of bacterial leaf spot, have been reported on sunflower in several places worldwide; symptoms are usually brown spots surrounded by a chlorotic halo. In older leaves, the lesions coalesce forming large irregular areas (Figure 4H) and end as dead tissue (Saharan et al. 2005; Arsenijevic 1994), these symptoms have also been observed in the infected plants of this study.

A. alternata causes leaf spot, the most common foliar disease on sunflower cultivars and its' symptoms are chlorotic and necrotic leaves, as it was observed on the infected plants of this study (Figure 4A, Figure 4B, Figure 4C). It is known that this pathogen affects the plant mainly during its growing stages, destroying leaf tissue and reducing photosynthetic capacity (Lagopodi and Thanassoulopoulos 1998). *S. sclerotiorum,* the cause of white rot, shows variable symptoms depending on the plant host (Boland and Hall 2009). In sunflower vegetative stages, lesions are brown and extend upwards (Figure 4I) and white mycelia appear on the leaf surfaces (Figure 4F) (Saharan et al. 2005); similar to the lesions seen on the infected plants of this study.

The report of these diseases on sunflower is very valuable information for Ecuador given that bacterial leaf spot caused by *P. cichorii* and white rot diseases caused by *S. sclerotiorum* have not been previously recorded in this country, neither some pathovars of *P. syringae.* Identification of bacterial pathovars is useful in order to classify species for

pathogenicity (Vicente et al. 2001); however this was out of the scope of this study and was not determined for any of the bacterial species. The national agricultural authorities "Agencia Ecuatoriana de Aseguramiento de la Calidad Agro" and "Instituto Nacional de Investigaciones Agropecuarias" have not registered the presence of *P. syringe* in Ecuador up to date, although *P. syringae* pv. phaseolicola has been prevously reported as affecting Ecuadorian *Phaseolus vulgaris* cultivars (Peralta et al. 2010). Therefore its presence cannot be disregarded and these studies can contribute to alerting the authorities on this respect. On the other hand, according to Agrocalidad 2014, *A. alternata* has been found in crops such as corn, beans, strawberries and tomatoes in Ecuadorian cultivars. This information will be useful for effective control measures, which depend mainly on a proper identification of the pathogen (Riley et al. 2002), and also the control of these diseases can contribute for sunflower production in the country.

In respect to the results obtained in this study, it is also important to mention that bacterial leaf spot and white rot detected in sunflower can spread to other plant species. For instance, *P. syringae* has a record of infecting stone fruits, mango, pear and peach cultivars (Kennelly et al. 2007) and *P. cichorii* can infect garlic, celery, cauliflower, watermelon, carrot, peach, tomato, lettuce, corn and banana cultivars according to CABI 2012. On the other hand, *S. sclerotiorum* can affect legumes, stone fruits, banana and most vegetables (Heffer and Johnson 2007). All these crops are found in Ecuador and some of them have great economic importance such as banana cultivars, one of the five most important products in Ecuadorian economy (BCE 2016).

This study was done with plants from only one commercial sunflower farm so it is highly recommended to analyze additional farms to determine the prevalence of these diseases and other potentially susceptible crops that could be affected.

The treatment and control of these sunflower diseases are essential as is the need for new antimicrobial compounds due to the rapid development of microbial resistance to existing drugs (Tejesvi et al. 2012). The search for alternative biopesticides such as plant and endophyte extracts is a promising option. Endophytic fungi have been recognized as a rich source of various secondary metabolites, which have beneficial activities as antimicrobial, insecticidal, cytotoxic and anticancer compounds (Aly et al. 2011l; Schulz et al. 2002; Silva et al. 2006; Forcina et al. 2015; Patridge et al. 2015). In this study, the antimicrobial activity of several endophytic fungi extracts was tested and bactericide activities in

CEQCA-O3215e2 extracts against both pathogenic bacteria were found (*P. syringae* and *P. cichorii*); fungicide activity was found with CEQCA-O1113e1 extract against both pathogenic fungi (*A. alternata* and *S. sclerotiroum*) and CEQCA-O3215d1 extract showed bioactivity against *S. slcerotiorum*. Other extracts had, bactericide and fungicide activity, such as the extracts CEQCA-G1396e2, CEQCA-O3215d2, CEQCA-O32115e2 (Table 4),

The extracts obtained from the fungus CEQCA-O3215, was identified as *Fusarium* sp. which is an ubiquitous soil pathogen and a common endophytic fungi of many plants. This genus has many species registered as a rich source of biological metabolites such as *F. oxysporum, F. solani* and *F. moniliforme* which have antimicrobial bioactivity (Wang et al. 2011; Somwanshi and Bodhankar 2015; Nampoothiri et al. 2013).

In this study, minimal inhibitory concentrations were obtained for three of the extracts tested which showed bactericide activities against both species of bacteria, *P. syringae* and *P.cichorii*; CEQCA-O3215e2 was the most potent extract, since its MIC value was lower than the MICs of the other two extracts against both bacteria. All MICs obtained should be considered as a reference point for further studies since they were obtained from crude extracts and not purified compounds.

Additionally, plant extracts were also tested. Plants can also produce secondary metabolites and are a valuable source of new and biologically active molecules that posses antimicrobial effects (Gurjar et al. 2012), and serve as plant defenses against pathogenic microorganisms (Cowan 1999). This study showed that none of the plant extracts presented total inhibition against any of the sunflower pathogens tested, but extract BV415a1 showed partial inhibition against both pathogenic bacteria and extract BV439a1 against both pathogenic fungi. According to Nostro et al. 2001, the effectiveness of an antibacterial agent is measured by its ability to inhibit or kill the bacteria so it is recommended to test these extracts at a higher concentration to verify its antibacterial or antifungal activity.

The results previously described could be explained by the additive effects that several bioactive compounds have rather than arising from a single compound. Different bioactive compounds in a mixture, in this case as crude extracts, can interact to provide a combined effect (Ginsburg and Deharo 2011). However, this study suggests to conduct future studies that include purification of all extracts that presented inhibitory activities in order to

understand which biologically active compound or group of compounds are responsible of these activities and then, further develop these as biopesticides.

Another option as a treatment against phytopathogenic microorganisms is the use of endophytic fungi as antagonists (Ullah et al. 2011). In this study CEQCA-O3215 (*Fusarium* sp) and CEQCA-G1396 showed inhibition against both pathogenic bacteria and one pathogenic fungi, therefore, they were chosen for *in vivo* assays. Endophytes are obligate symbionts but even endophytes can became pathogenic to other hosts depending on the fungi, plant genotype and environmental conditions (Faeth and Fagan 2002). In this study, both chosen fungi endophytes infected all sunflower plants, therefore, it is recommended to work with CEQCA-O3215 and CEQCA-G1396 extracts, or to test another method of fungal colonization such as the application of microbial antagonists directly to the soil or media. This sort of treatment could be used in crop production to prevent or reduce diseases caused by soil borne pathogens (Ullah et al. 2011). The disease severity obtained with this suggested method could be compared with the disease severity obtained in this study.

Effects of the volatile organic compounds (*Muscodor* sp.) against sunflower pathogens showed their potential as fungicides and can be used against *P. syringae, P. cichorii* and *S. sclerotiorum*, but not against *A. alternata*. *Muscodor* fungi have biocidal properties due to its production of a mixture of volatile organic compounds that include alcohol, acids, esters, ketones, and aromatic hydrocarbons (Rundell et al. 2015). These compounds can be used as an alternative for chemical fumigants, lowering the use of chemical fungicides, which are hazardous for human health and environment (Alpha et al. 2015). It is suggested to analyze the activity of the volatile organic compounds of *Muscodor* sp. against plant diseases, reported here on sunflower, because understanding the mechanisms of action of volatile organic compounds will help establish the appropriate application method for industrial mycofumigants.

Plant and fungal endophytic extracts can be a promising source of biopesticides; secondary metabolites are biodegradable, easy to produce on a large scale and could be easily exploited industrially (Katoch et al. 2014). Further research should be done to purify its active compounds and identify their chemical structure in order to develop biopesticide application to control and treat diseases that can affect Ecuadorian flower production.

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Figure 6. Seed treatment with antagonist endophytes fungi *in vitro*. A: Endophyte fungi CEQCA-G1396 B: Endophyte fungi CEQCA-G03215. Arrows show: Necrotic spots (Dilution 2×10^{-2}), leaf deformation (Dilution 2×10^{-2} day 6), brow spots (Dilution 2×10^{-2} day 3), mycelia (Dilution 2×10^{-2} day 6), mycelia (Dilution 2×10^{-8} day 10).

Table 1. Disease Severity Index Criteria (DSI) Maselli et al. (2000)

Grade	Criteria
0	Absence leaf lesions
1	Spots of 3mm around inoculation site, with or without halo.
2	Spots of 3.1 to 5mm around inoculation site, with halo
3	Spots around inoculation site greater than 5mm with defined halo
4	Spots outside inoculation site (halo)
5	Converge withering leaf spot
6	Dead

Table 2. Organisms used and their extracts. It shows plant, endophyte extracts and VOCs producer fungus

Organism Code	Identification	Organism	Extract Code	Extraction Solvent	Concentration mg/ml
CEQCA-O1113	Marasmicaceae	Endophyte	CEQCA-O1113 e1	EtOAc [a]	8.36
CEQCA-M1319	-	Endophyte	CEQCA-M1319 e1	EtOAc	4.26
CEQCA-M1189	*Nigrospora oryzae*	Endophyte	CEQCA-M1189 e1	EtOAc	4.22
			CEQCA-M1189 d1	CH_2Cl_2 [b]	4.25
CEQCA-M1193	*Xylaria* sp.	Endophyte	CEQCA-M1193 d3	CH_2Cl_2	4.2
			CEQCA-M1193 d1	CH_2Cl_2	4.23
CEQCA-P0501	*Gliocladium* sp.	Endophyte	CEQCA-P0501 e1	EtOAc	8.4
CEQCA-M1242	*Xylaria laevis*	Endophyte	CEQCA-M1242 e1	EtOAc	4.22
CEQCA-M1226	Dothideomucetes	Endophyte	CEQCA-M1226 e1	EtOAc	4.25
CEQCA-M1214	*Entonaema pallida*	Endophyte	CEQCA-M1214 e1	EtOAc	4.26
CEQCA-O1096	-	Endophyte	CEQCA-O1096 e2	EtOAc	8.44
			CEQCA-O1096 d2	CH_2Cl_2	4.3

Table 2. Continuing

Organism Code	Identification	Organism	Extract Code	Extraction Solvent	Concentration mg/ml
CEQCA-M1273	-	Endophyte	CEQCA-M1273 d1	CH_2Cl_2	4.26
CEQCA-M1262	*Pezizomycotina* sp.	Endophyte	CEQCA-M1262 e2	EtOAc	4.22
CEQCA-O1074	*Chrysochlamys* sp	Endophyte	CEQCA-O1074 d2	CH_2Cl_2	4.24
			CEQCA-O1074 e1	EtOAc	4.21
			CEQCA-O1074 e2	EtOAc	4.23
CEQCA-G1396	-	Endophyte	CEQCA-G1396 e1	EtOAc	122.8
			CEQCA-G1396 d2	CH_2Cl_2	32.9
			CEQCA-G1396 e2	EtOAc	103
			CEQCA-G1396 m1	CH_3OH [c]	139.4
CEQCA-O3215	*Fusarium* sp.	Endophyte	CEQCA-O3215 d2	CH_2Cl_2	56
			CEQCA-O3215 e2	EtOAc	126.4
			CEQCA-O3215 m1	CH_3OH	135.2
			CEQCA-O3215 d1	CH_2Cl_2	20.1
CEQCA-G0003	*Muscodor* sp.	Endophyte	CEQCA-G0003 d1	CH_2Cl_2	5.34

Table 2. Continuing

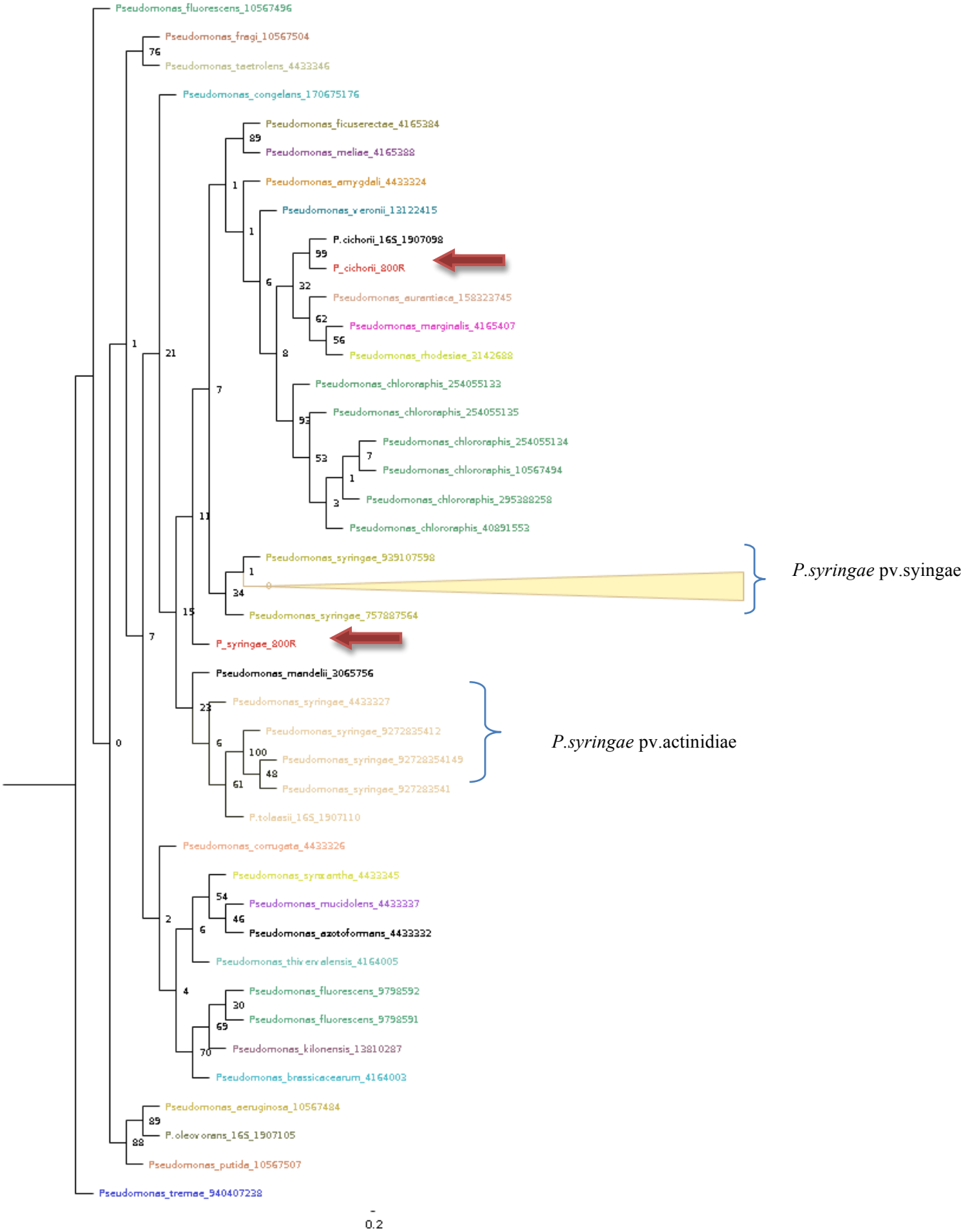
Organism Code	Identification	Organism	Extract Code	Extraction Solvent	Concentration mg/ml
BV407	*Pourouma bicolor*	Plant	BV407 1A	C_3H_6O [d]	38.8
BV415	*Bixa orellana*	Plant	BV415 1A	C_3H_6O	406
BV406	*Browneopsis ucayalina*	Plant	BV406 D1	CH_2Cl_2	89.8
BV400	*Piper* sp.	Plant	BV400 1A	C_3H_6O	64.3
BV436	*Attalea maripa*	Plant	BV436 1A	C_3H_6O	110
BV439	*Cecropia membranacea*	Plant	BV439 1A	C_3H_6O	868.3

Table 3.- MADI-TOF bacteria identification

Bacteria Code	Genus	Species	Spectra score
11A	*Pseudomonas*	*brassicacearum*	1.998
12A	*Pseudomonas*	*caripapayae*	1.829
31A	*Pseudomonas*	*putida*	2.154
43A	*Pseudomonas*	*cichorii*	1.938
21B	*Pseudomonas*	*chlororaphis*	1.939
42B	*Pseudomonas*	*savastanoi*	1.871
51B	*Pseudomonas*	*koreensis*	2.113
52B	*Pseudomonas*	*congelans*	1.85
42C	*Pseudomonas*	*syringae*	1.792
52C	*Pseudomonas*	*lutea*	1.891
42D	*Pseudomonas*	*thivervalensis*	1.749
21A	*Bacillus*	*mojavensis*	1.854
33A	*Bacillus*	*mycoides*	1.978
53A	*Bacillus*	*pumilus*	1.874
43B	*Bacillus*	*cereus*	1.805
52D	*Bacillus*	*siralis*	1.851
T1	*Stenotrophomonas*	sp.	1.866
52A	*Pantoea*	*agglomerans*	2.124
32A	*Paenibacillus*	*amylolyticus*	2.17
T2	*Ewingella*	*americana*	1.724

Table 4. Effective antibacterial and antifungal activities of endophyte fungi and plant extracts.

	Pseudomonas syringae		*Pseudomonas cichorii*		*Sclerotinia sclerotiorum*		*Alternaria alternata*	
Extract code	Effectivenes of Inhibition	Average size Halo (mm)	Effectivenes of Inhibition	Average size Halo (mm)	Effectivenes of Inhibition	Percent of inhibition (%)	Effectivenes of Inhibition	Percent of inhibition (%)
CEQCA-O1113 e1	-	-	-	-	+	81	+	36
CEQCA-O1096 e2	+	8	-	-	-	-	-	-
CEQCA-G1396 e2	++	14	++	13	+	74	++	100
CEQCA-G1396 e1	++	13	++	12	++	100	+	40
CEQCA-O3215 d1	-	-	-	-	++	100	+	64
CEQCA-O3215 d2	++	13	++	11	++	100	-	-
CEQCA-O3215 e2	++	13	++	14	++	100	-	-
CEQCA-M1193 d1	-	-	+	5	-	-	-	-
CEQCA-P0501 e1	+	5	-	-	-	-	-	-
BV439 a1	-	-	-	-	+	61	+	37
BV415 a1	+	17	+	16	-	-	-	-
BV400 a1	-	-	-	-	+	70	-	-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Pseudomonas_amygdali_4433324
Pseudomonas_veronii_13122415
P.cichorii_16S_1907098
P_cichorii_800R
Pseudomonas_aurantiaca_158323745
Pseudomonas_marginalis_4165407
Pseudomonas_rhodesiae_3142688
Pseudomonas_chlororaphis_254055133
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P.syringae pv.actinidiae

Figure 1.-

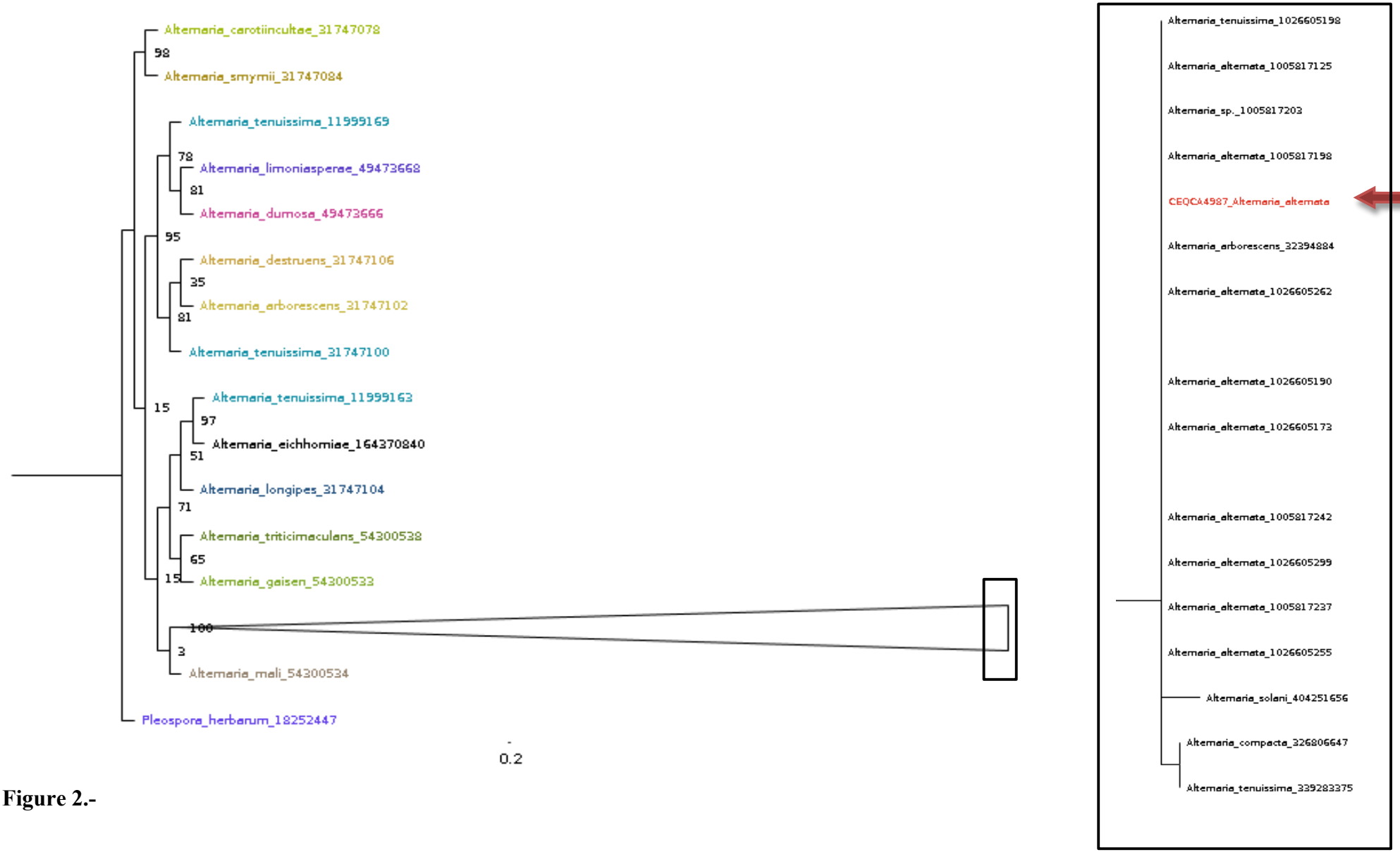
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Figure 2.-

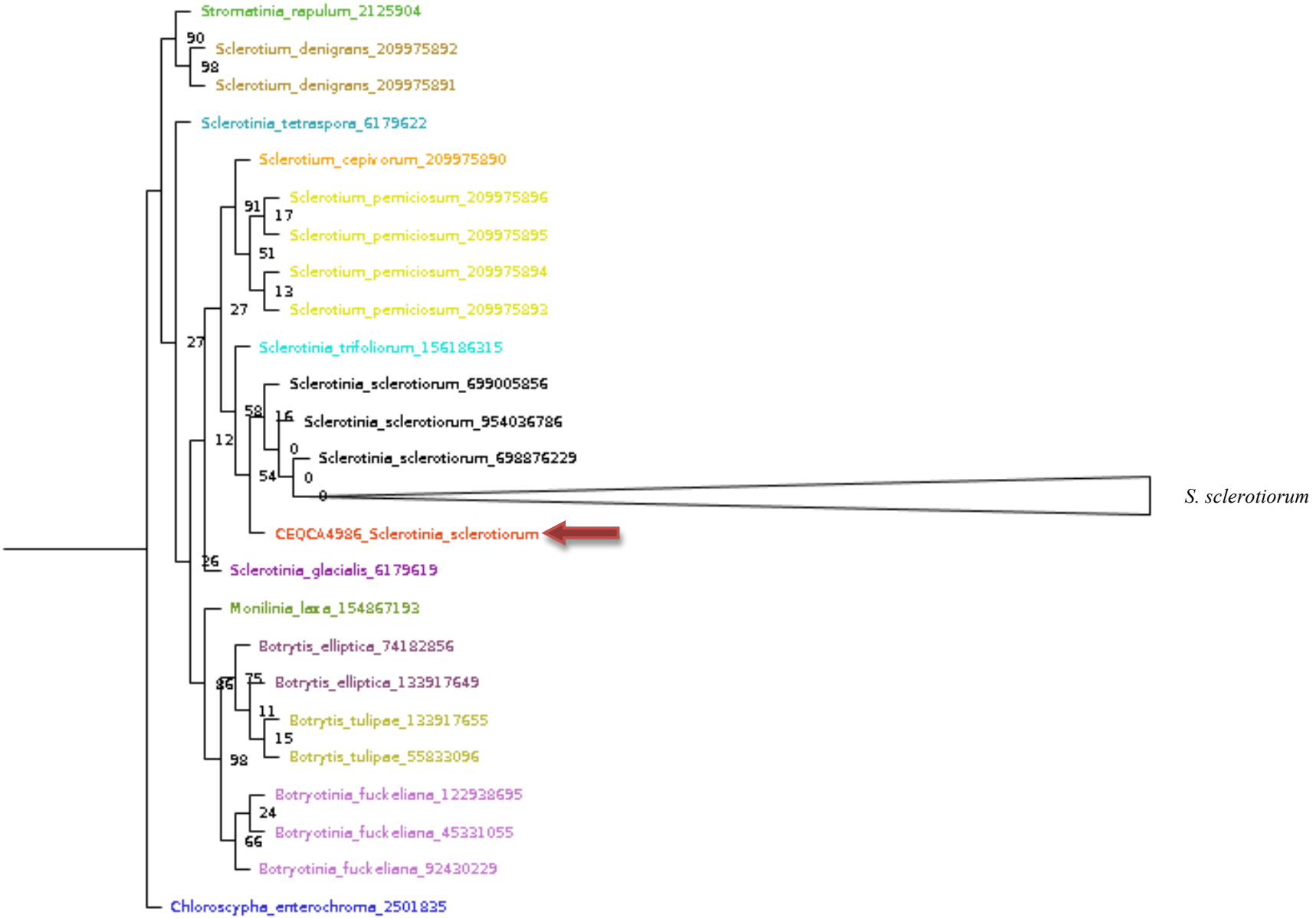
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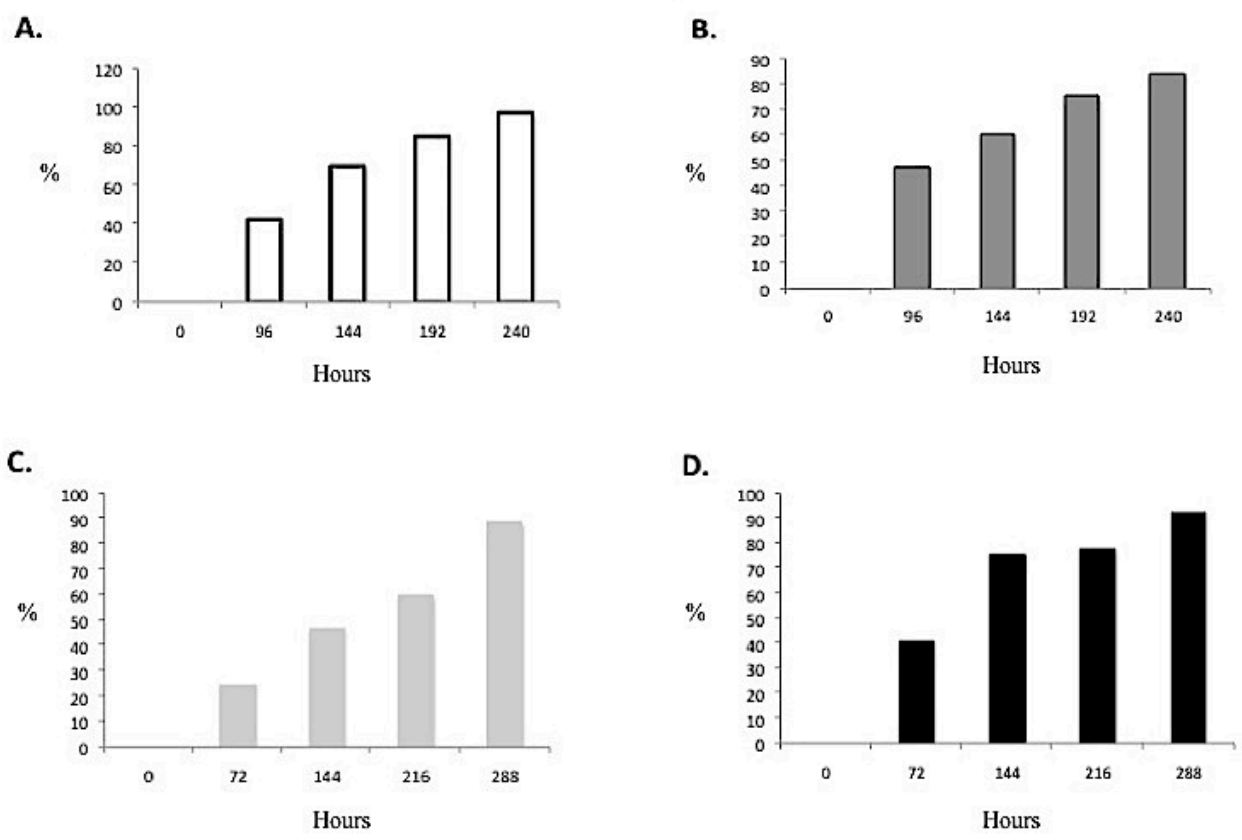
Figure 3.-

Figure 4.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Hours

Figure 5.

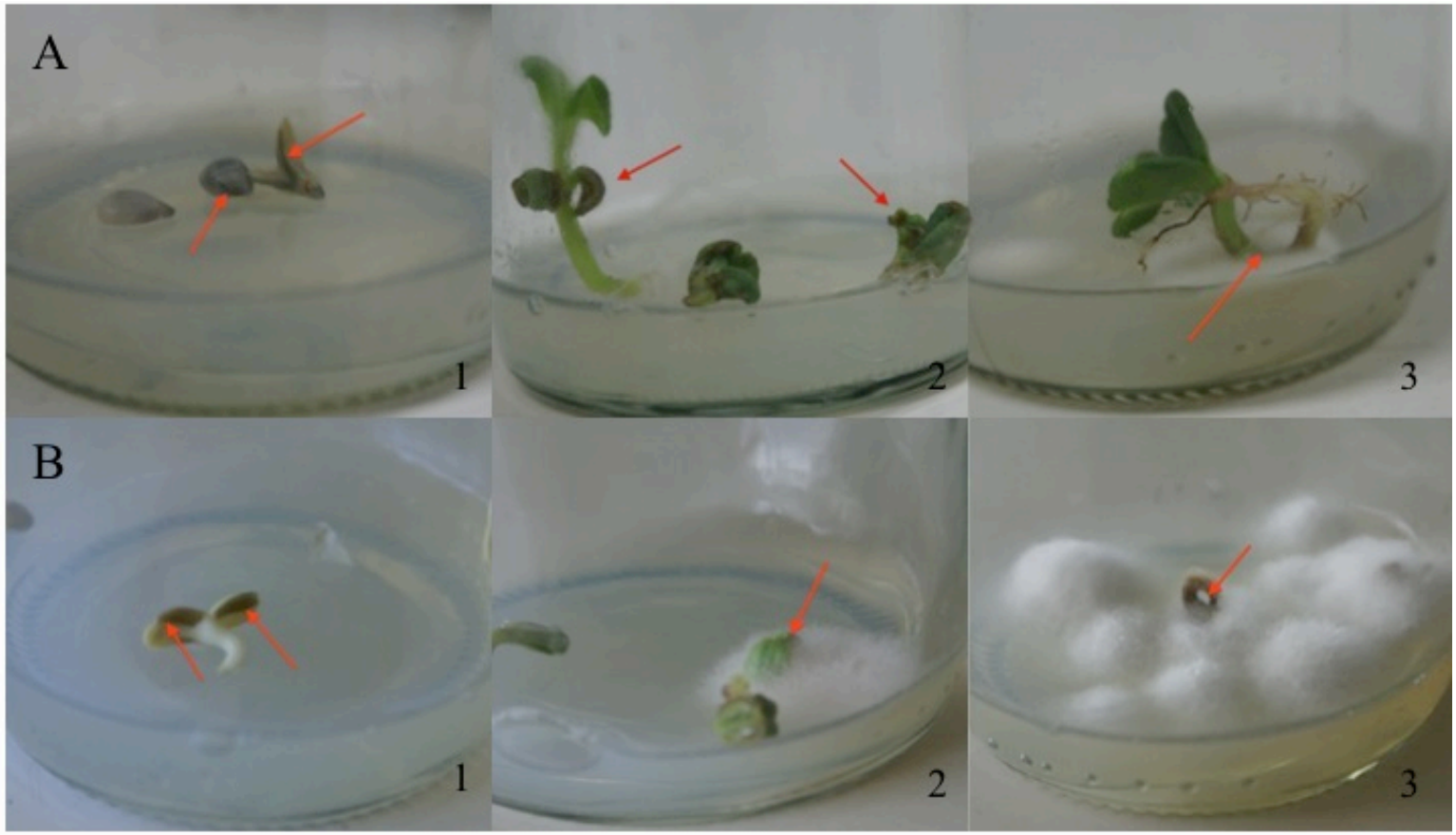

Figure 6.

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